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The purpose of these studies was to develop key data for the approach to the proof-of-principle, efficacy studies in Aim 5 using orthotopic Her-2/neu over-expressing human breast adenocarcinoma models.

Investigations pertinent to Aims 1 (apoptosis), 2 (formulation) and 4 (toxicity) were initiated and are still ongoing.

In two models examined to date, one model demonstrated interactions which were additive or greater between Wortmannin-mediated inhibition of the Akt/PKB pathway and sphingolipid-induced apoptosis. These studies were in related ovarian cell lines and are presently being extended to human breast adenocarcinoma lines, with basal or high Her-2/neu expression.

In formulation studies, we demonstrated that liposomes composed of 33 mole percent dimethyl-sphingosine (DMSp) and the balance of the lipids composed equivalently of dipalmitoyl-phosphatidyl choline and distearoyl-phosphatidyl choline, could be successfully formulated and that these were stable.

In toxicity studies, female nude mice tolerated well i.p. injections of as high as 4 mg equivalents of DMSp as small unilamellar vesicles (SUVs) of the composition described above. In related anti-tumor efficacy studies using liposomal-DMSp administered on Days 1 and 2, a dose of 3.75 mg DMSp equivalent per injection gave a T/C of ~108 in a human ovarian carcinoma model.

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Ann Westergaard 6/22/2000

PI - Signature Date

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Introduction

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In formulation studies, we demonstrated that liposomes composed of 33 mole percent dimethyl-sphingosine (DMSp) and the balance of the lipids composed equivalently of dipalmitoyl-phosphatidyl choline and distearoyl-phosphatidyl choline, could be formulated and were stable.

In toxicity studies, female nude mice tolerated well i.p. injections of as high as 4 mg equivalents of DMSp as small unilammelar vesicles (SUVs) of the composition described above. Preliminary studies using i.p.-implanted human ovarian tumor xenografts demonstrated marginal efficacy (T/C ~ 108) in response to two injections of 3.75 mg DMSp as SUVs.

Body

Task 1

We are currently finishing characterization of the responses of Her-2/neu basal-expressing (MDA-MB-231, MDA-MB-435, MDA-MB-468 and MCF-7) and high-expressing (BT-474, MDA-MB-453, SK-BR-3 and AU-565) human breast adenocarcinoma cell lines to C_6-ceramide, sphingosine, dimethyl-sphingosine and Wortmannin, as single agents. Dye-uptake viability assays are being employed. In the next phase we will combine each of the sphingolipids, at marginally toxic doses, with non-toxic levels of Wortmannin. This will determine whether inhibition of the Akt/PKB anti-apoptotic pathway will enhance the apoptotic response to these sphingolipids. Biochemical apoptotic endpoints of caspase-3 activation and PARP cleavage will be ascertained.

Task 2

Studies herein are pending completion of Task 1.

Task 3

Previously reported liposomal formulations of sphingolipids, including sphingosines, used for in vivo studies have mostly been composed at most 10 mole percent sphingolipid. Since we have in vitro evidence consistent with a monotonic relationship between increasing tumor cell killing by liposomal-sphingolipids and increasing sphingolipid mole percent, we conducted experiments to evaluate the feasability of markedly increasing the sphingolipid content. We were able to determine that small unilamellar vesicles (SUVs) composed of equimolar amounts of dimethyl-sphingosine (DMSp), dipalmitoyl-phosphatidyl choline (DPPC) and distearoyl-

phosphatidyl choline (DSPC), could be readily prepared by the lipid film approach. The only caveat we encountered was that the choice of solvent for dissolution of the DMSp should preferably be ethanol rather than DMSO, because of the difficulties in roto-evaporation of the latter in lipid film preparation. Otherwise, no difficulties were encountered in preparation, including subsequent aqueous dispersion and sonication, or in stability, compared to SUVs with lower sphingolipid content. These non-targeted SUVs were employed in the in vivo Tasks described below.

Liposomes which include sphingolipid and Wortmannin are pending formulation after obtaining compelling data in Aim 2.

Task 4

Long-circulating (PEG-containing) liposomes are pending formulation after completion of Aim 3.

Task 5

No further progress has been made on this Aim using BALB/c mice, but limited analysis has proceeded in nude mice (see Task 6).

Task 6

We had previously established that nude mice could readily tolerate 1.5 mg per dose of sphingosine as a liposomal (SUV) formulation of sphingosine/DPPC/DSPC (1:2:2), which was administered i.p. three times per week. Since there was little or no apparent toxicity from this treatment, and since previous studies had not reported MTDs for any liposomal-sphingolipid formulation, we conducted experiments to better define an MTD for the lead, non-targeted formulation.

We used SUVs composed of DMSp/DPPC/DSPC (1:1:1), and administered this formulation to female nude mice at 1, 2.5 and 4.0 mg per dose, giving two doses on consecutive days. Over five days, there was no weight loss for the 1 mg group, a slight (2%) weight loss for the 2.5 mg group, and a greater (5%) weight loss for the 4.0 mg group.

Although these studies were suggestive of some toxicity with the highest dose and that an MTD might be within range, we have not yet pushed the dose higher to firmly establish it. This dose and schedule were used in another study to examine efficacy using a human ovarian carcinoma xenograft model (see below).

Task 7

Studies with long-circulating (PEG-containing) liposomes are pending formulation after completion of Aims 3, 4 and more progress in 6.

Task 8

Based on Co-PI Dr. Mien-Chie Hung's experience with Her-2/neu over-expressing human breast adenocarcinoma orthotopic models, we attempted an initial efficacy experiment with non-targeted SUVs composed of DMSp/DPPC/DSPC (1:1:1), using MDA-MB-361 cells.

Two million cells were implanted in the mammary fat pad of female nude mice, and at the time of tumor outgrowth, i.v. administration of liposomes at the dose schedule described above was planned. For reasons not yet clear, unfortunately no tumor outgrowth occurred, even after several months.

The next experiments will employ SUVs administered at nearer the true MTD, and with a human breast adenocarcinoma tumor model which is more predictable in our hands.

In related studies using a human ovarian carcinoma model (HEY), we conducted a pilot study using modest doses of SUVs composed of DMSp/DPPC/DSPC (1:1:1). HEY cells were implanted i.p. on Day 0, and mice were segregated into four groups: 1) controls, 2) DMSp, administered as 1 mg free lipid in DMSO/PBS within 1 hr of injecting tumor, 3) liposomal-DMSp, administered on Days 1 and 2, 2.5 mg DMSp equivalent per injection, and 4) liposomal-DMSp, administered on Days 1 and 2, 3.75 mg DMSp equivalent per injection. Mice were sacrificed when they became moribund from tumor involvement. The results are shown in Table 1.

Table 1

Survival of Female Nude Mice Bearing i.p. HEY Tumors and Treated i.p. with Free and Liposomal-DMSp

	Controls	Free DMSp (1 mg)	L-DMSp (2.5 mg)	L-DMSp (3.75 mg)
	29[a], 29, 29	27, 27, 30	27, 29, 29, 29	27, 30, 34, 34
T/C	------------	96.6	98.3	107.8

a) Day of sacrifice due to morbidity from tumor progression

These results hint at an anti-tumor effect with increasing doses of DMSp. Although not part of the proposal, these studies will be pursued independently, as they may offer greater understanding of the basis for the anti-tumor efficacy of liposomal-sphingolipids.

Task 9

This Task will be undertaken once results from Task 8 have developed, allowing comparison of the efficacy of non-targeted and long-circulating SUVs.

Tasks 10 and 11

These will be undertaken when the corresponding mouse studies are well-advanced.

Task 12

These studies may be undertaken shortly, as ^{3}H-sphingosine which will be used as a tracer in the SUVs is now in hand.

Task 13

This will be undertaken when the corresponding studies with non-targeted SUVs are well-advanced.

Task 14

For obvious reasons, this will focus on the proof-of-principle, efficacy studies, and will not be done until the most promising anti-tumor efficacy data is defined.

Key Research Accomplishments

- Preparation of high-sphingolipid content SUVs
- Determination of increase in tentative MTD of DMSp-SUVs by ~ 0.5 log
- With sub-MTD protocol, demonstration of slight anti-tumor efficacy in i.p. tumor model

Reportable Outcomes

One manuscript is in preparation which will the initial report on the effects of Her-2/neu expression on apoptotic responses to sphingolipids.

Conclusions

A major conclusion is that the multiple-dose MTD for DMSp as non-targeted SUVs administered i.p. is > 4 mg. This is already much higher than used previously in murine models which demonstrated anti-tumor efficacy and will be pushed higher to more firmly establish the true MTD. This dose, assuming full bioavailability and its distribution in an estimated peritoneal volume of 1.0-1.5 ml, would achieve an intraperitoneal concentration of 8-12 mM, far higher (> 2 logs) than required to induce apoptosis in vitro in any tumor cell line we have examined. If the volume of distribution includes the plasma volume, the concentration would still likely be several millimolar, which is most relevant to systemic treatment.

Since i.v. and i.p. administration of liposomal-sphingolipids will be employed in the efficacy studies to systemically treat nude mice with orthotopically-implanted breast tumors, we wanted to first establish whether i.p. drug administration would demonstrate efficacy against an i.p.-implanted (regional) tumor. We observed only modest activity against an ovarian tumor model (HEY) with a sub-MTD protocol of non-targeted SUVs of DMSp/DPPC/DSPC, which suggests that pharmacokinetic studies will need to optimally guide the efficacy studies. Distribution of sphingolipids to hydrophobic non-target sites, e.g., plasma membranes, albumin, lipoproteins, could reduce their bioavailability, and causing deviation from the calculations cited above.

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